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Research report

Acute sleep deprivation enhances avoidance learning and spatial memory and induces delayed alterations in neurochemical expression of GR, TH, DRD1, pCREB and Ki67 in rats

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HIGHLIGHTS

- Our study assessed effects of acute versus repeated sleep deprivation periods.
- Acute SD was associated with improved learning and memory.
- Repeated SD led to elevated GR-ir at the PVN, CA1 and CA3 regions.
- Acute and repeated SD differentially regulated neurochemical signaling.
- SD effects on behavior and brain systems were found to be duration-related.

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ABSTRACT

The current study investigated the effects of acute versus repeated periods of sleep deprivation on avoidance learning and spatial memory and on the expression of discrete biochemical brain signals involved in stress regulation, motivation and brain plasticity. Male Long–Evans rats were sleep deprived using the platform-over-water method for a single 4 h period (ASD) or for daily 4 h RSD period on five consecutive days (CSD). The Y maze passive avoidance task (YM-PAT) and the Morris water maze (MWM) were used to determine learning and memory 1 h following the last SD period. Region-specific changes in glucocorticoid receptors (GR), tyrosine hydroxylase (TH), dopamine 1 receptors (DRD1), phospho-CREB (pCREB) and Ki-67 expression were assessed in the hippocampal formation, hypothalamus and mesolimbic regions 72 h following RSD. Behaviorally, our findings revealed increased latency to re-enter the aversive arm in the YM-PAT and reduced distance traveled and latency to reach the platform in the MWM in ASD rats compared to all other groups, indicative of improved avoidance learning and spatial memory, respectively. Acute SD enhanced TH expression in the ventral tegmental area, nucleus accumbens and A11 neurons of the hypothalamus and DRD1 expression in the lateral hypothalamus. Cell proliferation in the subventricular zone and pCREB expression in the dentate gyrus and CA3 regions was also enhanced following acute SD. In contrast, repeated SD significantly elevated GR-ir at the hypothalamic paraventricular nucleus and CA1 and CA3 layers of the hippocampus compared to all other groups. Our study supports that a brief 4 h sleep deprivation period is sufficient to induce delayed neurochemical changes.

1. Introduction

Sleep deprivation (SD) has shown negative effects on cognitive function in both humans and animals [1,2]. Various animal studies indicate that extended periods of sleep deprivation (SD) lead to memory impairments in several behavioral tests including the Morris water maze [3,4], radial maze [5,6] as well as discriminative and passive avoidance tasks [7–10]. However, studies assessing shorter-term sleep deprivation (<24 h) have generated mixed findings, showing no effect [7] or improved memory retrieval [11] in passive avoidance tasks.

In parallel to behavioral assessments, recent findings indicate altered expression of neurochemical signals associated with activity-dependent plasticity following sleep deprivation. Thus,

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upregulation of brain-derived neurotrophic factor (BDNF) and TrkB receptors have been observed following short (8 h) periods of sleep deprivation [12]. In contrast, 20 days of 'gentle touch' sleep deprivation in mice led to reduced expression of phosphorylated cyclic AMP responsive element binding protein (pCREB) in the hippocampus, an important transcription factor of learning and memory-related plasticity [13,14]. Consistent with these observations, extended periods of SD have been associated with reduced cell proliferation [15,16] and neurogenesis [17] in the dentate gyrus. More recently, sleep deprivation has also been shown to affect neurochemical signaling in discrete regions of the mesolimbic system. For example, using the platform-over-water paradigm [17], 72 h SD led to 15% decrease in dopamine type 1 receptors (DRD1) density in the mice striatum [18]. Differences in chronically SD versus stressed animals suggest that changes affecting dopaminergic signaling are not merely a by-product of stress [18]. In this regard, a study by Hanlon and colleagues [19] indicated that although rats in a five-day SD condition showed comparable palatable food gain as non-deprived rats during the initial 3–6 min of a 15 min test, these rats showed reduced sucrose pellet consumption in the remaining testing time, suggesting that they remembered how to accomplish the task, but motivation appeared diminished. Nonetheless, stress has well-established effects on dopamine secretion and receptor dynamics (for reviews see [20,21]) and glucocorticoid release regulates appetitive/motivational behaviors, in part via modulation of meso-accumbens dopamine neurotransmission [22,23]. Increased corticosterone secretion following SD has also been associated with impaired hippocampal LTP [24]. Noteworthy, it was recently demonstrated [25] that while 72 h SD impaired memory retrieval, a shorter period rather facilitated memory retrieval, an effect mimicked by acute administration of synthetic corticosterone and prevented by administration of the glucocorticoid synthesis inhibitor metyrapone. Interplay of these systems thus likely contributes to SD effects.

At present, effects of short and longer term sleep deprivation period have for most been observed in separate studies, making comparisons of duration-related behavioral and neurochemical changes difficult due to the use of different rodent species and/or strains and sleep deprivation paradigms. The first objective of the current study therefore aims to characterize effects of a single versus repeated daily 4 h sleep deprivation period(s) on passive avoidance in the Y maze, and spatial learning and memory in a delayed non-matching to sample version of the Morris Water Maze. A second objective aims to assess alterations in the expression of markers of cell proliferation (Ki-67) and plasticity-related gene expression (pCREB), glucocorticoid receptors (GR), as well as tyrosine hydroxylase (TH) and dopamine receptor 1 (DRD1) expression in the mesolimbic pathway 72 h following sleep deprivation. These findings will enable a more direct comparison of the behavioral and neurochemical impact of acute and repeated periods of SD.

2. Materials and methods

2.1. Animals

Fifty adult male Long–Evans rats (300–350 g) were obtained from Charles River Laboratory (Rochefort, Quebec, Canada). Upon arrival, animals were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 am), room temperature (21–23 °C) with 60% relative humidity, and ad libitum access to water and standard Purina rat chow. Rats habituated to the animal facility for one week prior to SD. All procedures were carried out in accordance with the Canadian Council of Animal care and approved by the University of Ottawa Animal Care Committee.

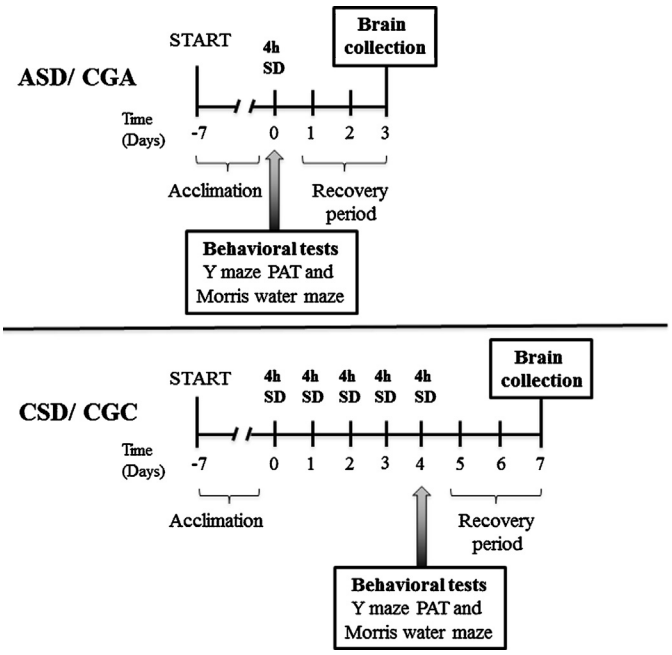

Fig. 1. Timeline for the acute and chronic conditions (top and bottom panels, respectively). Rats in the ASD group were SD for a single 4 h period, while rats in the CSD group were SD for a 4 h period for 5 consecutive days. Control groups (CGA and CGC) were placed in identical chambers as experimental rats but on larger platforms. A home cage group (HC) not exposed to SD acted as controls. Rats were killed 3 days following behavioral testing.

Animals were randomly divided into five groups ($n = 10$/group). Acute (ASD) and chronic (repeatedly) sleep deprived (CSD) rats acted as the experimental groups, and both had respective control groups (CG) named CGA and CGC, respectively. A group of naïve animals (home cage group (HC)) provided an additional control in behavioral and post-mortem assessments, as well as to control for the equally stressful conditions of the small and large platforms.

2.2. Sleep deprivation

The ASD and CSD groups were sleep deprived at light onset. ASD rats were exposed to a single 4 h period, while CSD rats were repeatedly sleep deprived for 4 h per day on five consecutive days. Rats in the control groups (CGA and CGC) were exposed to the same environmental conditions as experimental groups with the exception of the availability of a bigger platform in the sleep deprivation boxes (Fig. 1). Sleep and slow-wave activity has been shown maximal in the beginning of the 12 h light period in rodents [26], and sleep deprivation at this time period, even for short durations (ranging from 3 to 6 h), exerts significant effects on sleep pattern recovery [27,28], and cognition using the same SD paradigm [29,30].

The classical platform-over-water paradigm (also called the 'inverted flower pot' technique) was used, as previously described [17,30,31]. Briefly, large opaque plastic containers (63.5 cm L × 38 cm W × 48 cm H) were filled with 5–7 cm of water, and a small (5 cm in diameter) or a larger (20 cm in diameter) platform was secured over an inverted clay flowerpot placed in the center of the container. Three walls of the container were covered with dark blue cardboard, allowing the rat to partially see their environment, but preventing them from seeing rats of other groups being tested at the same time. Experimental groups were placed on small platforms allowing all sleep stages except paradoxical (REM) sleep. REM sleep stage-associated muscle atonia caused the rats to fall off the small platform into the shallow pool of water, forcing them to climb back up onto the small platform, and stay

awake. It has been suggested that using a weight to platform area ratio (10:1 in experimental groups and 1:1 in control groups; [3]) in determining a suitable size for the platforms would serve as an adequate control for the platform technique [7,32,33]. This, according to Mendelson and colleagues [32], would mean that the animal would undergo a greater reduction in REM sleep when the platform is smaller relative to its size. Other studies have used narrow platforms ranging in diameter from 6 cm to 7 cm in the experimental groups and 11.5 cm to 14 cm in the control groups [7,34–36]. EEG-controlled studies have supported the validity of the platform paradigm in preventing REM sleep (see [17,37–39]) and the model has traditionally been used to characterize effects of REM sleep deprivation. Recent evidence however suggested that the procedure is accompanied by reduced time spent in NREM sleep stages [40]. Body temperature variation associated with the procedure in the first rat cohort demonstrated that readings fell within the normal body temperature range for rats and the procedure was discontinued.

2.3. Behavioral testing

Behavioral testing began immediately following the last SD period (between 11 am and 12 pm). Rats were transported to distinct testing rooms outside (YM-PAT) or inside (MWM) the vivarium. After transportation to a novel room, rats were allowed to habituate in their home cages a minimum of 30 min prior to testing. The YM-PAT was conducted before the MWM and this sequence was applied for all animals.

2.3.1. Y Maze passive avoidance task (YM-PAT)

The Y Maze (35.5 cm L × 15 cm W × 30 cm H) was used to assess the rats' retention of an aversive stimulus by measuring the latency to re-enter the arm paired with the aversive stimulus on a second maze exposure. This paradigm was designed based on previous studies using air-puffs to induce aversive learning [41,42]. Rats were initially placed in the maze and allowed to freely explore either arm. Latency to enter the first arm (L1) was recorded in seconds. Once the rat entered one arm of the maze with its four paws, a sliding door was gently closed behind him and the rat then received small jets of compressed air (air puffs) applied directly to its face every 15 s over a 60 s period before being removed from the maze. Use of the air puff as an aversive stimulus has been validated as an alternative to foot shock in passive avoidance paradigms [41]. A 2-min inter-trial interval was applied before re-exposure to the maze. Upon the second exposure, the arm paired with the aversive stimuli was the only one available for entry. Latency to reenter this arm (L2) was measured in seconds. Maximal latency for re-entry into the aversive arm was 5 min. The apparatus was cleaned with 70% ethanol and dried in-between testing for each animal.

2.3.2. Morris Water Maze (MWM)

The Morris water maze (MWM) consisted of a white circular pool (176.5 cm in diameter, 45 cm high) filled with water (35 cm depth) and divided into four quadrants. The pool was located in a dimly lit room and was surrounded by black curtains on which posters and calendars served as extra-maze cues in each direction (N, S, E, W). The water was kept at a constant temperature of 21 °C and rendered opaque by the addition of non-toxic white paint hiding the platform and allowing easy detection of the trajectory (distance traveled) of Long Evans rats. The Plexiglas circular escape platform (15 cm in diameter with an adjustable height) was submerged 5.08 cm below the water surface. Individual behavior was monitored using a video camera suspended above the pool and a computerized tracking system (Ethovision 3.1, Noldus IT). The experimenter sat in the corner of the testing room facing a computer screen, which monitored a live feed of the pool, and recorded latency to reach the platform with a stopwatch.

A delayed alternation version of the MWM task was selected as it represents a more complex MWM task previously shown to be sensitive to SD effects [29], and which involved switching the platform's location (left or right from the starting point) between each trial. Rats received a habituation trial with the platform placed in the middle, followed by twelve test trials with a cutoff time of 1 min to find the platform and an inter-trial interval of 10 min. All rats were placed on the south side of the pool and the platform was alternated between the east and west locations for the subsequent trials. The dependent variables included latency and distance traveled to reach the platform. A 13th trial took place with the platform removed. This probe trial assessed learning by measuring swim latency in the quadrants paired with the platform versus the unpaired quadrants.

2.4. Brain tissue preparation

Three days following behavioral testing, rats were killed with an overdose of Euthansol (120 mg/kg; i.p.) and their brain rapidly extracted, frozen on dry ice and stored at −80 °C until sectioning. Coronal brain sections (14 μm) were collected on polarized Superfrost® Plus slides (Fisher Scientific, Canada) using a cryostat (Leica CM1900, Leica Microsystems, Germany) and stored at −80 °C until processed. Brain tissue was collected for the different regions of interest according to coordinates from the Paxinos and Watson rat brain atlas (1998): nucleus accumbens and lateral hypothalamus (NAc, LH ~ Bregma −1.00 mm), paraventricular nucleus of the hypothalamus (PVN ~ Bregma −1.30 to 2.12 mm), hypothalamic A11 region, subventricular zone (SVZ) of the lateral ventricles, dentate gyrus (DG), (CA1 & CA3 layers of the hippocampus ~ Bregma −2.80 to −4.16 mm), and ventral tegmental area (VTA ~ Bregma −5.60 mm).

2.5. Immunohistochemical detection

A subset of animals (n = 6/group) was used for immunohistochemical detection of glucocorticoid receptors (GR, raised against amino acids 121–420 mapping within the internal region of GRα of human origin), tyrosine hydroxylase (TH, purified from PC12 cells, recognizes an epitope on the outside of the regulatory N-terminus), dopamine-1 receptor (DRD1, raised against a peptide mapping at the C-terminus of DRD1 of human origin), Ki-67 (raised against a human Ki67 nuclear antigen expressed in all proliferating cells during late G1, S, S2, G2 and M phases of the cell cycle), and phosphoCREB (pCREB, recognizes p43 phosphorylated CREB, and may also recognize p30 and p38 kDa proteins). The antibody specificity information was derived from the manufacturers' data sheet. Brain sections were post-fixed for 5 min in 4% PFA in 0.1 M phosphate buffered saline (PBS; pH = 7.4), after which they were rinsed 3 × 5 min in 0.01 M PBS. Brain sections were then incubated with the distinct primary antibodies diluted in a PBS solution containing 98% PBS/2% Triton X-100. Primary antibodies concentrations and incubation conditions were as follow: rabbit polyclonal antibody for GR (1:500, sc-8992, SantaCruz Biotechnology) and mouse monoclonal antibody for TH (1:1000, MAB318, Millipore Corporation) were incubated at 4 °C for 24 h; goat polyclonal antibody for DRD1 (1:400, sc-1434, SantaCruz Biotechnology) incubated at 4 °C for 60 h, mouse monoclonal antibody for Ki-67 (1:200, NCL-L-Ki67-MM1, NovoCastra Labs) incubated for 1 h at room temperature, and mouse monoclonal antibody for phosphoCREB (1:400, 06-519, Millipore Corporation) incubated for 48 h at 4 °C. Following primary antibody incubation, sections were rinsed 3 × 5 min in PBS and incubated at room temperature with the proper Alexa 488-conjugated secondary antibody: 2 h

incubation with the donkey anti-rabbit IgG (1:500, Invitrogen), donkey anti-mouse IgG (1:500, Invitrogen), donkey anti-goat IgG (1:500, Invitrogen) and donkey anti-mouse IgG (1:500, Invitrogen) and 30 min incubation for the donkey anti-mouse IgG (1:400, Invitrogen). The Alexa fluor-conjugated secondary antibodies were diluted in the blocking solution previously described. Special controls omitting the primary antibodies were also performed in order to determine non-selective binding. Following three rinses, slides were incubated with 1 μg/ml (Hoechst 33342, Invitrogen Canada Inc) for 10 min at room temperature to label cell nuclei. An antifade medium containing 0.1% *p*-phenylenediamine in phosphate buffered glycerol was then applied and the slides coverslipped and sealed with nail polish. Signal detection was accomplished using an Olympus DX51 microscope (Center Valley, PA, USA). Digital images of immunofluorescence were obtained using the Progress Pro 2.7.6 software under 20× magnification. Brain regions were selected as part of biochemical systems that are shown to mutually influence each other [35,43–45]. As SD has sometimes been argued to represent a biological stressor, inclusion of regions and biochemical markers where stress effects has been documented allowed us to investigate whether sleep deprivation differs in its neurochemical signature. For all regions of interest, the staining intensity of GR, TH, DRD1 and pCREB immunoreactive cell bodies or processes were quantified using Image J software (National Institutes of Health, Bethesda, MD) and the method described by Hayes and colleagues [46]. Percentages of optical densities (mean gray values: estimates of intensity of the tissue stain) from a selected brain region relative to a subthreshold background were obtained. This technique is required to initially subtract the background and then measure intensely labeled area. Four anatomically matched pictures of the left and right hemispheres of the brain were used to produce an average immunoreactivity score for each brain region in each animal. Data are presented as background corrected standardized image densities for each brain area.

2.6. Quantification of Ki-67 labeling

Immuno-positive cells for Ki-67 ($n = 6$ per group) in bilateral hemispheres of the subgranular zone (SGZ) of the DG, and the subventricular zone (SVZ) of the lateral ventricles were manually counted using an Olympus BX51 fluorescence microscope at 40× magnification. Briefly, a cell was located in the subgranular zone if it was within 2 cell nucleus widths from the border between the granular cell layer and the hilus [47]. Labeled cells in the hilus were also counted, but separately since proliferating cells in this region are predominantly destined to become glia [47,48]. For cell counts in the SVZ, a cell was determined to be in the SVZ if it was within 8 cell nucleus widths from the outer walls of the lateral ventricles [47]. As there were three brain tissues per slide, each slide consisted of tissues collected at every 9th section (i.e. at 252 μm intervals; we collected a slice and skipped the next one, etc.). The mean number of bilateral hemispheric immunoreactive cells per slide was calculated for each of the animals, and then their group means were derived.

2.7. Statistical analysis

Statistical analysis was performed using SPSS Statistics (version 19). The assumptions of homogeneity of variance and Mauchly's sphericity were verified. The Huynh–Feldt correction for violations to the assumption of sphericity was applied when appropriate and the degrees of freedom adjusted. Y Maze data was analyzed using a mixed ANOVA with two factors – sleep (RSD versus non-RSD) and duration (acute versus chronic) – and time as the repeated factor (Latency 1 and 2). A similar analysis was performed for the distance traveled and latency in the MWM with trials (12 in total) being the repeated factor. Simple main effects analysis with Bonferroni adjustment at critical alpha level (.05) was used to further explore the significant interactions. One-way ANOVA followed by Tukey's post hoc comparisons served to assess between group differences (to include the HC group) for distance and latency scores. For the MWM probe trial, a one-way ANOVA was used followed by Tukey's post hoc comparisons.

Separate two-way ANOVAs were performed to analyze the immunohistochemical data [optical density values or labeled cell numbers] for the different brain regions. Bonferroni corrected simple effect tests were applied when significant interactions were detected. The HC control group was not included in the initial two-way ANOVA analyses as it created an unbalanced design. When main effects or interactions were detected, one-way ANOVAs including the HC group were conducted to assess between group differences followed by Tukey's post hoc comparisons. For all analyses, $p < .05$ was considered statistically significant. Values are presented as means ± standard error of the mean (SEM). Pearson's product–moment correlation was performed to determine the relationship between immunohistochemical markers and behavior and between the different markers.

3. Results

3.1. Behavioral testing

3.1.1. Y maze passive avoidance test

Two-way repeated-measures ANOVA revealed a main effect of duration ($F(1,34) = 6.087$, $p = .019$) and time ($F(1,34) = 29.47$, $p < .001$). A significant duration × time interaction ($F(1,34) = 4.475$, $p = .042$) was found but no sleep × duration or sleep × duration × time interaction present. Simple main effect analyses revealed that duration of SD affected the latency to re-enter the aversive arm ($F(1,34) = 6.557$, $p = .015$) for the acute [Wilk's Lambda = 0.529, ($F(1,34) = 30.237$), $p < .001$] and chronic [Wilk's Lambda = .868, $F(1,34) = 5.184$, $p = .029$] conditions. One-way ANOVA on re-entry latencies revealed an effect of group ($F(4,45) = 4.891$, $p = .002$) attributable to increased latency to reenter the aversive arm in the ASD as compared to CSD ($p = .014$), CGC ($p = .027$) and HC ($p = .002$) groups, but not the CGA ($p = .122$). No between group differences was present upon the first maze entry (latency 1) (see Fig. 2).

3.1.2. Distance traveled and latency in the Morris Water Maze

Fig. 3 (top panels) shows the distance traveled by the different groups for the twelve trials. Two-way repeated-measures ANOVA revealed a main effect of sleep (($F(1,34) = 9.576$, $p = .004$), trials ($F(11, 24) = 6.106$), $p < .001$) and significant sleep × duration ($F(1, 34) = 7.251$ $p = .011$) and sleep × trials ($F(9.503, 323.109) = 2.53$, $p = .007$) interactions. Simple main effect analyses revealed that the sleep × duration interaction was due to altered distance traveled in the acute condition ($F(1,34) = 13.587$, $p = .001$). One-way ANOVAs revealed significant between group differences on Trials 2 ($F(4,45) = 3.987$, $p = .008$), 4 ($F(4,45) = 3.512$, $p = .015$), 6 ($F(4,45) = 2.907$, $p = .033$) and 11 ($F(4,45) = 3.445$, $p = .016$), attributable to reduced distance traveled by the ASD animals to reach the hidden platform.

Fig. 3 (bottom panels) shows the latencies to reach the platform over successive trials for the different groups. Two-way repeated measures ANOVA indicated a main effect of sleep ($F(1,36) = 4.181$, $p = .048$), duration ($F(1,36) = 5.577$, $p = .024$), trial ($F(11,396) = 8.357$, $p < 0.001$) and a sleep by trial interaction ($F(11,396) = 2.861$, $p < .001$). Reduced latencies over the successive trials was found for the sleep deprived [Wilk's Lambda = 0.245, ($F(11,26) = 7.298$), $p < .001$] and non-deprived [Wilk's Lambda = 0.110,

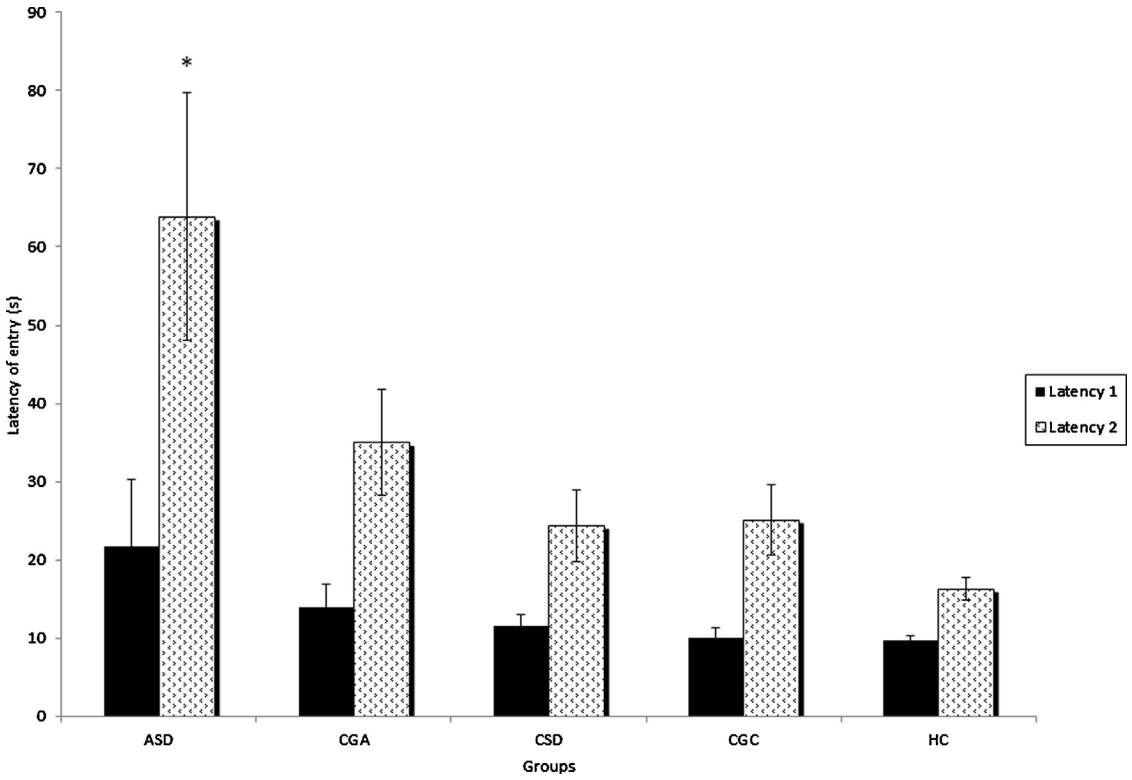

Fig. 2. Effects of sleep deprivation on passive avoidance in the Y-Maze. ASD group showed increased latency to re-enter the aversive arm compared to all other groups. CSD animals showed similar performance as control animals. Results are presented as mean latencies ± SEM. *$p < 0.05$.

F (11,26) = 19.111, $p < .001$] groups. One-way ANOVA revealed between group differences on Trials 1 (F (4,41) = 3.959, $p = .008$), 2 (F (4,41) = 19.470, $p < .001$), 3 (F (4,41) = 4.734, $p = .003$), 4 (F (4,41) = 3.923, $p = .008$), and 7 (F (4,41) = 5.378, $p = .001$). Tukey's post hoc comparisons indicated that these were attributable to reduced latency to reach the platform by the ASD group on the aforementioned trials. Fig. 4 depicts the time spent in the four quadrants – platform paired (1 & 3) versus unpaired (2 & 4) – during the probe trial (13th trial). Two-way repeated measures ANOVA indicated a main effect of quadrants (F (1,35) = 5.230, $p = .028$) and

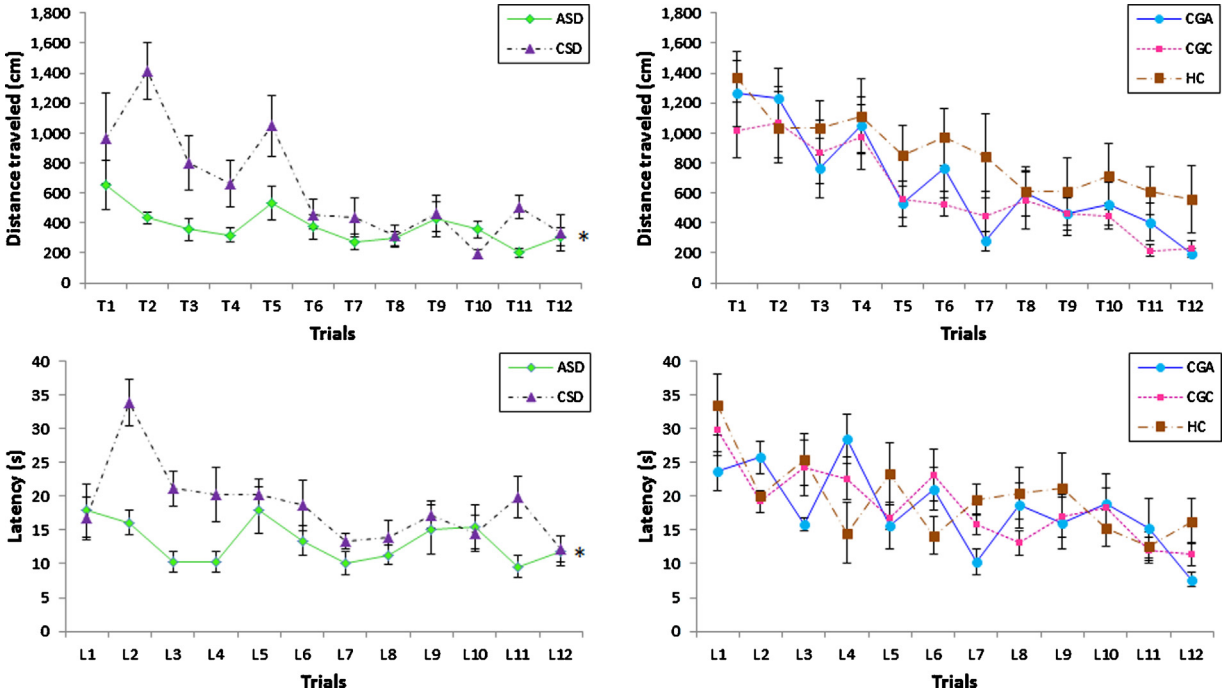

Fig. 3. Effects of sleep deprivation on distance traveled and latency to find the hidden platform in the Morris Water Maze. Distance traveled and Latency (in seconds) for the different groups in the twelve trials for the different groups in the twelve trials (top and bottom panels, respectively)-. Overall, the ASD group showed reduced distance traveled to reach the hidden platform. Results are presented as mean ± SEM. *ASD significantly different from all other groups ($p < 0.05$).

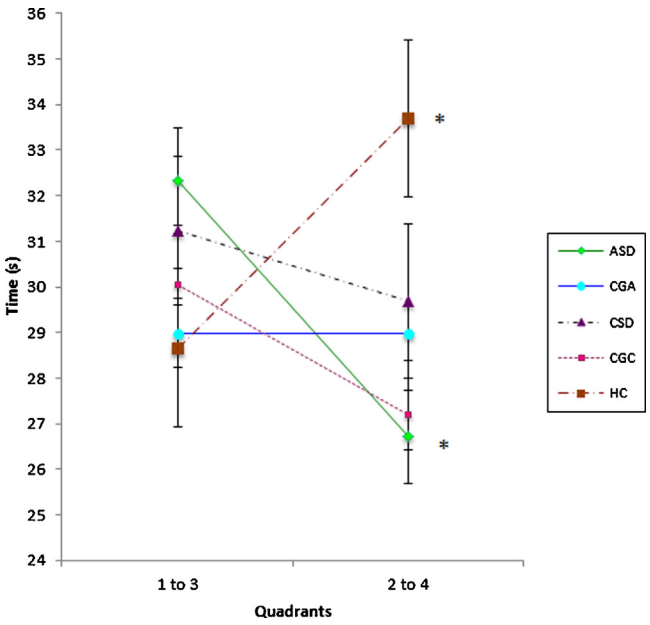

Fig. 4. Sleep deprivation effects on swim latency in the platform paired versus platform unpaired quadrants in the Morris Water Maze during the probe trial. Quadrants 1 & 3 were paired with the platform while quadrants 2 & 4 were not paired with the platform in the preceding twelve trials. The ASD group showed significantly increased time spent swimming in the paired compared to unpaired quadrants, suggesting enhanced learning of the alternation task. Results are presented as means ± SEM. $^{*}p < 0.05$.

sleep ($F(1,35) = 6.721$, $p = .014$) but no interactions between factors. Oneway ANOVA on the platform paired and unpaired quadrants during the probe trial indicated significant between group differences for the platform unpaired (2 & 4) quadrants ($F(4,44) = 4.814$, $p = .003$), with significant differences attributable to the HC group when compared to the ASD ($p = .003$) and the CGC ($p = .008$). One way ANOVA performed on swim time in each quadrant revealed between group differences for quadrant 1 ($F(4,44) = 3.303$, $p = .019$) and quadrant 2 ($F(4,44) = 5.084$, $p = .002$), attributable to increased swim time in ASD animals in quadrant 1, and increased swim time in HC animals in quadrant 2. There was no evidence of quadrant preferences in the other rat groups, which swam comparable times in the platform paired and unpaired quadrants.

3.2. Effects of SD on GR-ir expression

Two-way ANOVA performed on GR-ir within the nuclei of the CA1 pyramidal cell layer revealed main effects of sleep (F (1,14) = 24.921, $p < .001$), duration (F (1,14) = 43.792, $p < .001$) and a significant sleep × duration interaction (F (1,14) = 5.758, $p = .031$). Simple main effect analyses revealed that Sleep condition did not affect GR-ir expression in the acute condition (F (1,14) = 3.781, $p = .072$), while differences between sleep deprived and non-deprived animals were found in the chronic condition (F (1,14) = 24.586, $p < .001$). Duration influenced GR-ir expression for animals in the sleep deprived (F (1,14) = 40.654, $p < .001$) and non-sleep deprived ($F(1,14) = 8.896$, $p = .010$) conditions. One-way ANOVA comparing density values for the five groups also revealed between groups differences [F (4,17) = 32.33, $p < .0001$] (Fig. 5b). Tukey's post hoc comparisons indicated that GR-ir at the CA1 in the CSD group was significantly higher than that of all the other groups ($p < .001$). Significant differences in GR-ir were also detected between the CGC and the CGA, HC and CSD groups ($p < .05$), and the HC and ASD groups ($p < .01$).

Within the nuclei of the CA3 pyramidal cell layer, two-way ANOVA revealed a main effect of sleep (F (1,14) = 13.311, $p = .003$), duration (F (1,14) = 129.892, $p < .001$) and a significant sleep × duration interaction (F (1,14) = 12.041, $p = .004$). Simple main effect analyses revealed a significant effect of Sleep that was restricted to the chronic condition (F (1,14) = 22.802, $p < .001$). Of interest, duration (acute versus repeated) affected GR-ir expression for sleep deprived ($F(1,14) = 110.514$, $p < .001$) and non-sleep deprived (F (1,14) = 31.418, $p < .001$) animals. One-way ANOVA revealed significant between group differences [F (4,17) = 68.32, $p < .0001$] (Fig. 5c), attributable to significant differences between the CSD group and all other groups ($p < .001$). The CGC also significantly differed from the CGA, HC, ASD, and CSD groups ($p < .001$). Finally, the HC group significantly differed from the ASD group and CGA ($p < .05$).

Two-way ANOVA assessing the values of GR-ir at the parvocellular neuroendocrine cells of the PVN revealed a main effect of sleep (F (1,14) = 23.637, $p < .001$), duration ($F(1,14) = 149.126$, $p < .001$) and a significant sleep × duration interaction ($F(1,14) = 8.952$, $p = .010$). Simple main effect analyses indicated that sleep deprivation had no effect in the acute condition (F (1,14) = 1.967, $p = .183$), but significantly influenced GR-ir levels for animals in the chronic condition (F (1,14) = 27.757, $p < .001$). Duration influenced GR-ir levels in both the sleep deprived ($F(1,14) = 115.576$, $p < .001$) and non-sleep deprived (F (1,14) = 42.502, $p < .001$) conditions. One-way ANOVA combining all the groups revealed significant between group differences [$F(4,17) = 70.78$, $p < .001$] (Fig. 5d). Tukey's post hoc comparisons revealed that the CSD rats differed significantly from all the other groups ($p < .001$). Similarly, the CGC group differed significantly from all the groups ($p < .001$).

3.3. Effects of SD on TH- and DRD1-ir expression

Two-way ANOVA assessing TH dopamine expression at the VTA revealed a main effect of sleep (F (1,16) = 64.953, $p < .001$), duration (F (1,16) = 85.12, $p < .001$) and a significant sleep × duration interaction ($F(1,16) = 76.376$, $p < .001$). Simple main effects analyses revealed that effects of Sleep were limited to animals in the acute condition (F (1,16) = 161.380, $p < .001$). Duration influenced TH-ir in sleep deprived animals only (F (1,16) = 141.099, $p < .001$). One-way ANOVA revealed significant differences between the groups [F (4,20) = 60.34, $p < .0001$] for TH-ir levels (Fig. 6b), attributable to heightened TH-ir in the ASD compared to all other groups ($p < .0001$). The HC also had increased TH-ir compared to all groups except the ASD ($p < .0001$).

Within the nucleus accumbens dopamine neurons (NAc), analysis revealed no effect of sleep (F (1,16) = 3.635, $p = .075$), but a main effect of duration (F (1,16) = 32.194, $p < .001$), TH-ir expression being higher in the acute (M = 40.40, SD = 18.08) than for the chronic (M = 15.56, SD = 9.22) groups. Analysis also indicated a significant sleep × duration interaction ($F(1,16) = 21.805$, $p < .001$). Simple main effect analyses revealed that Sleep influenced TH-ir levels in the acute condition ($F(1,16) = 21.623$, $p < .001$), but not for animals in the chronic condition ($F(1,16) = 3.817$, $p = .068$). Duration influenced TH-ir for sleep deprived animals ($F(1,16) = 53.495$, $p < .001$), but had no effect in non-sleep deprived condition. One-way ANOVA revealed significant between group differences [F (4,20) = 20.87, $p < .0001$] (Fig. 6c), attributable to heightened TH-ir in ASD and HC rats compared to all other groups ($p < .0001$).

Within the hypothalamic A11 dopamine neurons, two-way ANOVA revealed a main effect of sleep (F (1,17) = 124.913, $p < .001$), duration (F (1,17) = 120.126, $p < .001$) and a significant sleep × duration interaction (F (1,17) = 145.215, $p < .001$). Simple main effect analyses indicated that Sleep influenced TH-ir in the acute condition (F (1,17) = 282.008, $p < .001$) and that duration had an effect only in sleep deprived animals (F (1,17) = 253.716, $p < .001$). Similarly, one-way ANOVA revealed between group differences [$F(4,20) = 74.34$, $p < .0001$] (Fig. 6d). Post hoc comparisons

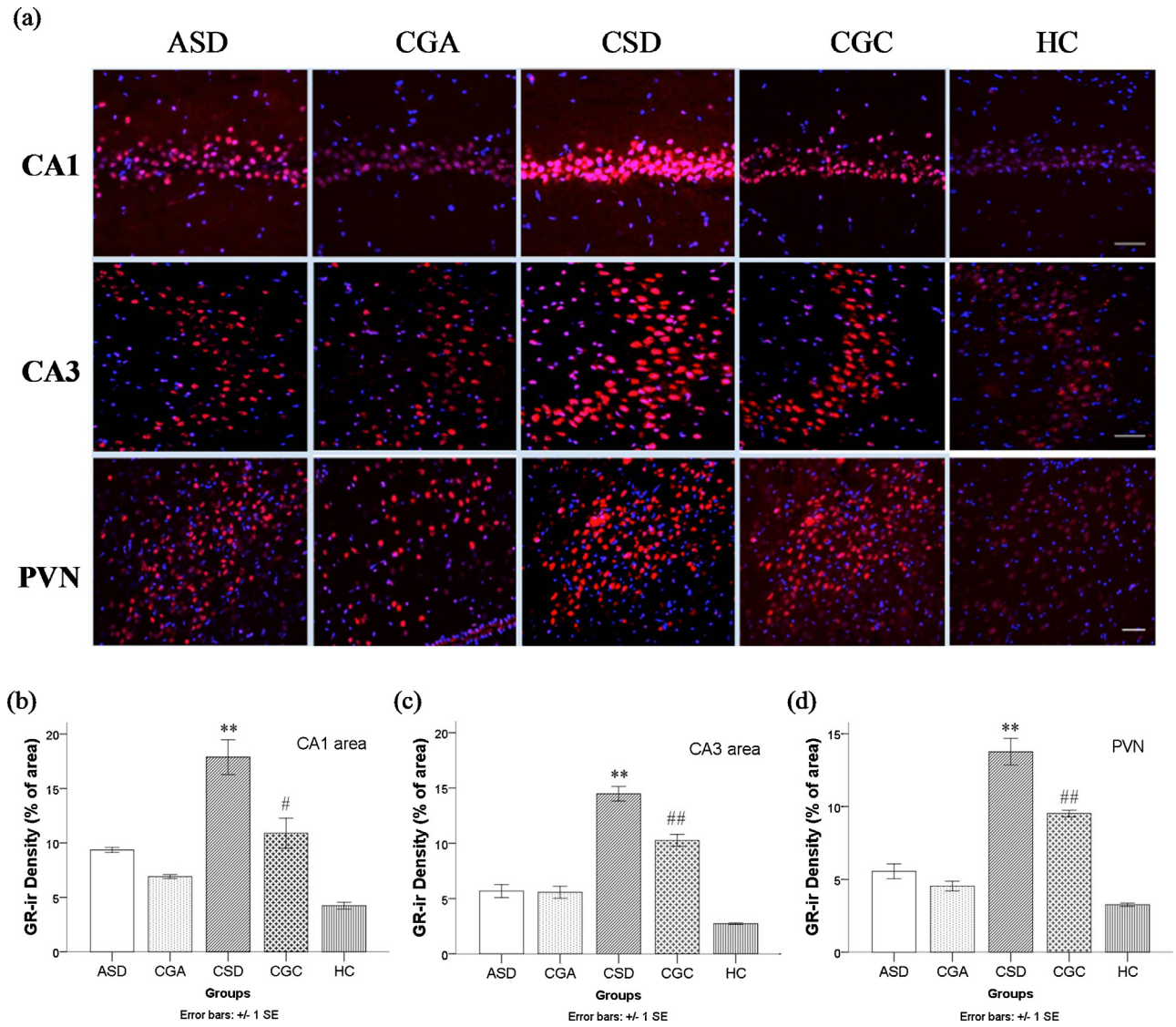

Fig. 5. Effects of sleep deprivation on GR-ir at the hypothalamic PVN and hippocampal CA1 and CA3 subregions. (a) Representative photomicrographs of GR-ir (red) combined with Hoescht (blue) labeling for the different groups. Scale bar, 50 μm (20× magnification). (b–d) Histograms showing GR-ir densities for the different groups. Results are presented as means ± SEM. Significantly different from ** all groups $p < 0.01$. # CGA, HC and CSD, $p < 0.05$. ## All groups except CSD, $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

showed that this was due to increased expression in the ASD compared to all other groups ($p < .0001$). HC also differed from that of the CGA and CSD groups ($p < .05$).

Fig. 7 depicts DRD1-ir expression and density values within the lateral hypothalamus. Two-way ANOVA revealed a main effect of sleep (F (1,16) = 33.740, $p < .001$), duration (F (1,16) = 72.359, $p < .001$) and a significant sleep × duration interaction (F (1,16) = 72.617, $p < .001$). Simple main effect analyses indicated that Sleep significantly affected DRD1-ir in the acute condition (F (1,16) = 104.816, $p < .001$) and that duration effects were significantly affected only in sleep deprived animals (F (1,16) = 161.450, $p < .001$). Finally, one-way ANOVA indicated significant between group differences [F (4,20) = 74.34, $p < .0001$] attributable to increased DRD1-ir in the ASD compared to all other groups ($p < .0001$).

3.4. Effects of SD on cell proliferation (Ki-67-ir)

Fig. 8 shows Ki-67 expression (found to be present in all active phases of the cell cycle, but not in the resting phase) and optical density values for all groups in the SVZ (dorsal region) of the lateral ventricles and dentate gyrus. In the SVZ, two-way ANOVA revealed a main effect of duration (F (1,12) = 8.758, $p = .012$) and a significant sleep × duration interaction (F (1,12) = 5.559, $p = .036$). Simple main effect analyses revealed that Sleep significantly affected Ki-67-ir of animals in the acute condition (F (1,12) = 7.063, $p = .021$) and that Duration had a significant impact on sleep deprived animals (F (1,12) = 14.137, $p = .003$). One-way ANOVA revealed significant differences between the groups (F (4,15) = 4.14, $p = .019$). Tukey's post hoc comparisons revealed that this was due to increased Ki-67 expression in the ASD compared to the CSD and CGC group ($p < .05$). However, within the dentate gyrus, two-way ANOVA revealed no effects of sleep (F (1,12) = 0.022, $p = .887$), duration (F (1,12) = 0.432, $p = .524$) and no sleep × duration interaction (F (1,12) = 0.186, $p = .674$).

3.5. Effects of SD on expression of plasticity-related protein (pCREB-ir)

Fig. 9 shows pCREB-ir located in the nuclei of the CA1 and CA3 pyramidal cell layers and in the dentate granule cells of DG of the hippocampus. For the dentate gyrus, two-way ANOVA revealed no

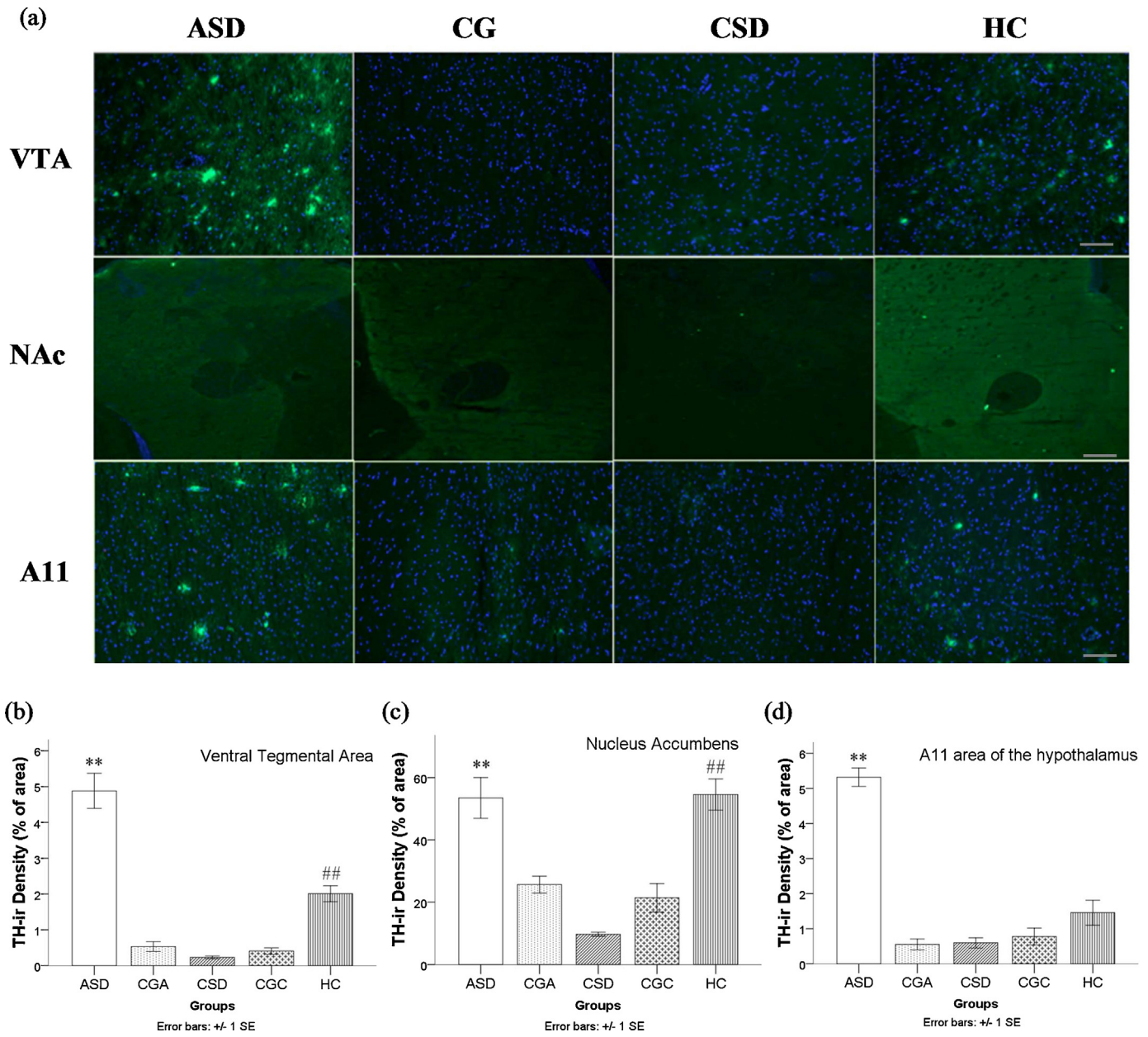

Fig. 6. Effect of sleep deprivation on TH-ir in the VTA, NAc shell, and A11 region of the hypothalamus. (a) Representative photomicrographs of TH-ir (green) and Hoescht (blue) labeling for the different groups. Scale bar, 50 μm (20× magnification). (b–d) Histograms showing TH-ir densities for each group. Results are presented as means ± SEM. VTA – **significantly different from all other groups, ##all groups except ASD, $p < 0.01$. NAc shell – **, ##from all groups but not from each other, $p < 0.01$. A11 – **from all groups, $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

effect of sleep ($F(1,15) = 0.300$, $p = .592$), but a main effect of duration ($F(1,15) = 8.930$, $p = .009$) and a significant sleep × duration interaction ($F(1,15) = 25.472$, $p < .001$). Simple main effects analyses revealed that effects of sleep influenced levels in both the acute and chronic conditions ($p < .001$). Duration effects were related to changes observed in the sleep deprived condition ($F(1,15) = 30.490$, $p < .001$). One-way ANOVA revealed between group differences [$F(4,18) = 9.55$, $p < .001$]. Tukey's HSD post hoc comparisons revealed that this was due to heightened pCREB-ir in the ASD compared to all groups ($p < .001$), except CGC. CSD rats showed reduced expression compared to CGC ($p < 05$) (Fig. 9b). For the CA1 region, analysis revealed no a main effect of duration ($F(1,15) = 7.694$, $p = .014$) only. One-way ANOVA revealed no differences between groups at that brain region [$F(4,18) = 1.94$, $p = .147$] (Fig. 9c). At the CA3, two-way ANOVA revealed a main effect of duration ($F(1,15) = 15.117$, $p = .001$) but no effect of sleep or sleep × duration interaction. One-way ANOVA indicated between group differences [$F(4,18) = 3.96$, $p = .018$] (Fig. 9d) attributable to increased pCREB-ir in the acute condition (ASD and CGA groups) compared to the CSD group ($p < .05$).

3.6. Pearson's correlation

A Pearson product–moment correlation coefficient was computed to assess the relationship between the immunohistochemical markers and behaviors. These analyses showed that increased pCREB-ir in the DG was negatively correlated with latency in the MWM ($r = -.467$, $p = .025$), while time to enter the YM-PAT on trial 2 was positively correlated with pCREB-ir in the CA1 ($r = .435$, $p = .038$) and in the CA3 ($r = .525$, $p = .010$) pyramidal layers. Pearson's product–moment correlation coefficient was also used to analyze the relationship between the different immunohistochemical

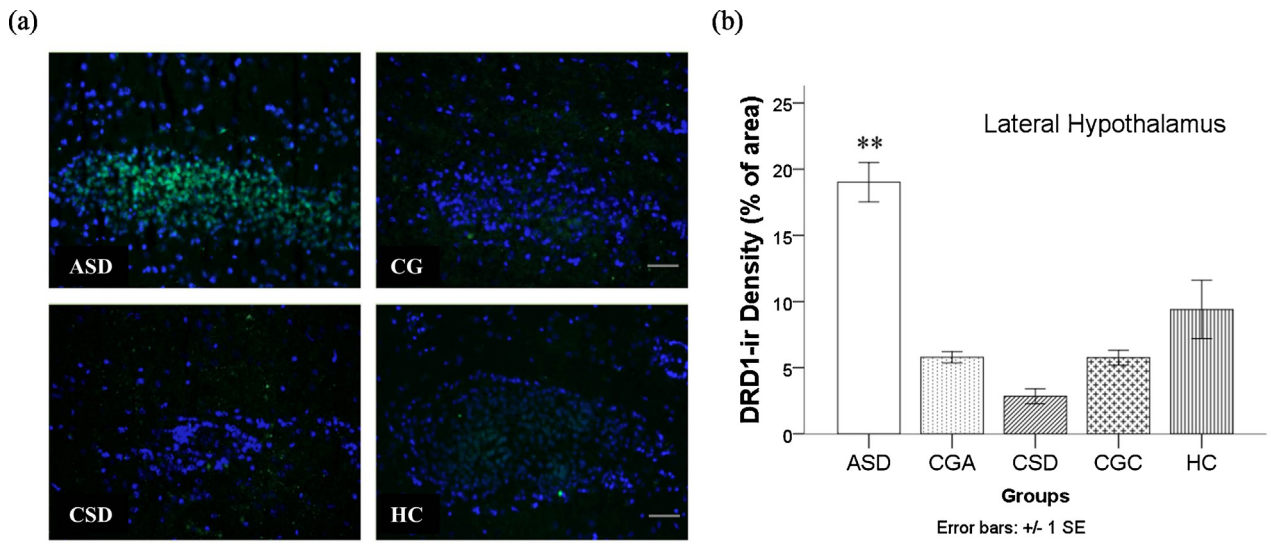

Fig. 7. Effect of sleep deprivation on Dopamine receptor 1 (DRD1) immuno-reactivity within the lateral hypothalamus. (a) Representative photomicrographs of DRD1-ir (green) and Hoescht (blue) labeling for the different groups. Scale bar, 50 μm (20× magnification). (b) Histogram showing DRD1-ir densities for all groups. Results are presented as means ± SEM. **Significantly different from all other groups, $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

markers. A negative correlation was found between GR-ir in the CA3 layer and TH-ir in the VTA ($r = -.469$, $p = .028$). There were strong negative correlations between GR-ir at the CA1 ($r = -.570$, $p = .006$), CA3 ($r = -.722$, $p < .0001$) and PVN ($r = -.703$, $p < .0001$) and TH-ir in the NAc. GR-ir in the CA1 ($r = -.434$, $p = .043$), CA3 ($r = -.556$, $p = .007$), and PVN ($r = -.572$, $p = .005$) was also negatively correlated with pCREB-ir in the CA3 layer. DRD1-ir in the LH was also negatively correlated with GR-ir in the PVN ($r = -.509$, $p = .016$) and the CA3 layer ($r = -.577$, $p = .005$). In general, results suggest an inverse relationship between GR-ir and dopamine markers. Finally, Ki67-ir in the SVZ was positively correlated with pCREB-ir in the CA3 ($r = .479$, $p = .033$) and DG ($r = .565$, $p = .009$), suggesting a link between these two plasticity-related markers in response to SD. A table summarizes these results (Table 1).

4. Discussion

The current study characterized the effects of single versus repeated 4 h SD period(s) on spatial navigation and avoidance learning, as well as changes in the expression of discrete brain signals regulating stress, motivation and brain plasticity. Our findings indicate improved spatial working memory in the MWM and Y-maze avoidance learning in acutely sleep deprived animals. At the neurochemical level, we observed differential and region-specific changes in GR-, DRD1-, TH-, pCREB-, and Ki67-ir following acute and repeated SD.

4.1. Acute SD period has beneficial effects on learning and memory

Behavioral testing initiated 1 h following SD indicated increased latency of ASD rats to re-enter the aversive (air-jet coupled) arm of the Y maze compared to all other groups, suggesting that short deprivation promoted the retention of an aversive emotional experience. Heightened arousal and/or stress-induced facilitation of memory formation could play a role in these observations as acute stress has been shown to enhance associative learning in rodents [49]. The latter proposition is supported by retention

Table 1
Pearson's product–moment correlation coefficient table of immunohistochemical markers with behaviors (A) and between the different markers (B).

A		Behaviors	
Markers	Regions of interest	MWM – Latency	YMPAT – Latency 2
PCREB	DG	$r = -.467^{*}$, $p = .025$, $n = 23$	$r = .305$, $p = .157$, $n = 23$
	CA1	$r = -.226$, $p = .299$, $n = 22$	$r = .435^{*}$, $p = .038$, $n = 23$
	CA3	$r = -.351$, $p = .100$, $n = 23$	$r = .525^{*}$, $p = .010$, $n = 23$

B		GR		
Markers	Regions of interest	CA1	CA3	PVN
TH	VTA	$r = -.249$, $p = .263$, $n = 22$	$r = -.469^{*}$, $p = .028$, $n = 22$	$r = -.383$, $p = .079$, $n = 22$
	NAc	$r = -.570^{**}$, $p = .006$, $n = 22$	$r = -.722^{**}$, $p < .0001$, $n = 22$	$r = -.703^{**}$, $p < .0001$, $n = 22$
DRD1	LH	$r = -.375$, $p = .086$, $n = 22$	$r = -.577^{**}$, $p = .005$, $n = 22$	$r = -.509^{*}$, $p = .016$, $n = 22$
PCREB	CA3	$r = -.434^{*}$, $p = .043$, $n = 22$	$r = -.556^{**}$, $p = .007$, $n = 22$	$r = -.572^{**}$, $p = .005$, $n = 22$

Markers	Regions of interest	PCREB	
		CA3	DG
Ki67	SVZ	$r = .479^{*}$, $p = .033$, $n = 20$	$r = .565^{**}$, $p = .009$, $n = 20$

* $p \leq 0.05$.
** $p \leq 0.01$.

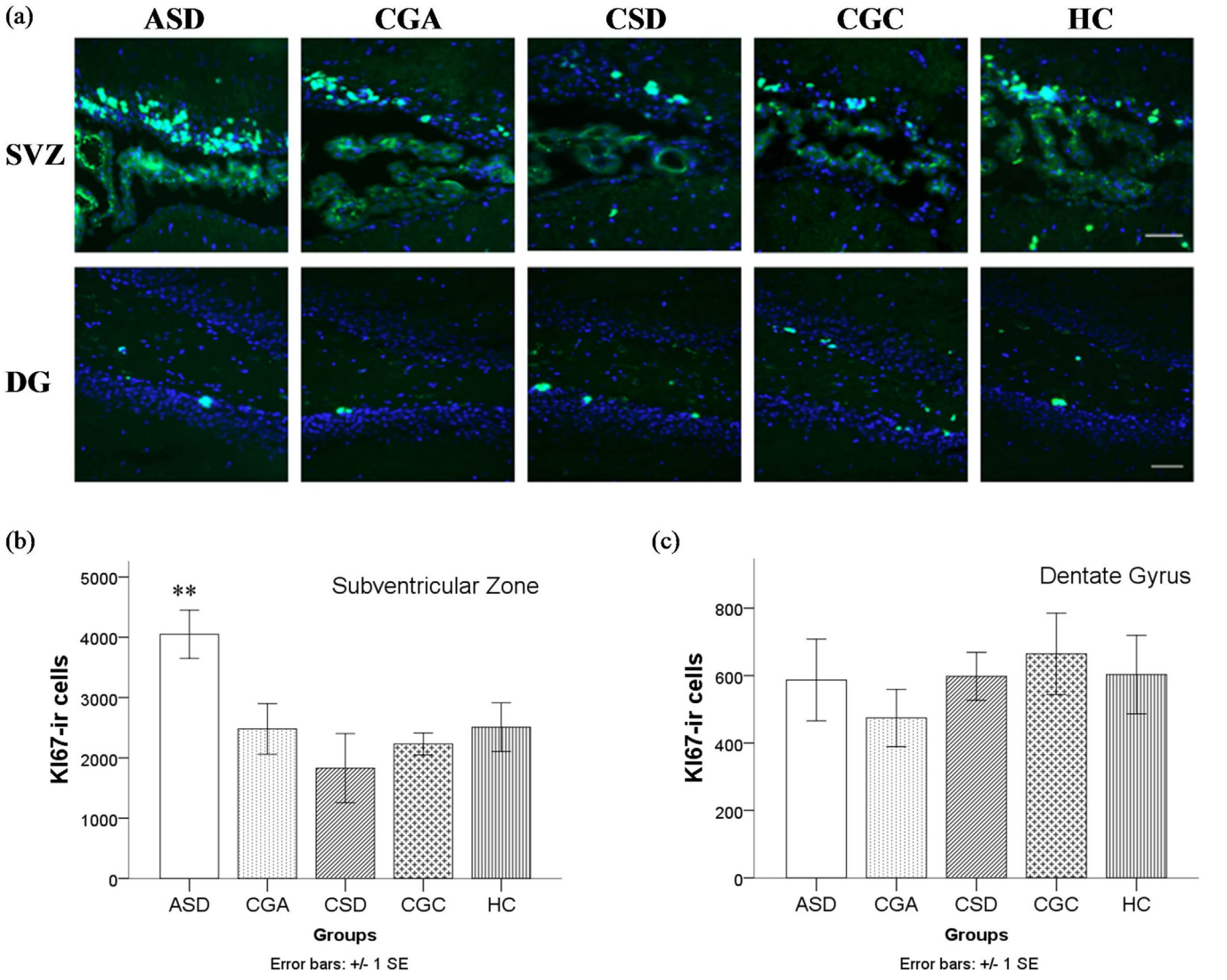

Fig. 8. Effect of sleep deprivation on KI67-ir in the subventricular zone and DG. (a) Representative photomicrographs of Ki67-ir (green) and Hoescht (blue) labeling for the different groups. Scale bar, 50 μm (20× magnification). (b and c) Histogram showing the mean Ki67-ir positive cells for each of the groups. Results are presented as means ± SEM. **Significantly different from CSD and CGC groups, $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

deficits observed in an avoidance learning task in rats injected with metyrapone prior to SD [25]. A trend toward increased latency to re-enter the aversive arm in the acute control animals in our study suggests a role for stress and/or heightened arousal in memory facilitation following ASD. Interestingly, sleep deprivation has been proposed to bring about some of its effects by activating the locus coeruleus noradrenergic (NA) system, a phenomenon shown to be associated with sensitized postsynaptic NA receptors in critical neuroplastic circuits. Such phenomenon would facilitate arousal and sharpened attentional processes [50]. Repeated bouts of 4 h SD over 5 days had no impact on avoidance learning, suggesting that animals adapted to this condition possibly via sleep rebound over the rest of the day [27,28,51]. This is supported by impaired associative learning in rodents exposed to continuous SD regimen for 72–96 h in the same paradigm [9,52]. Under such conditions, Silva and colleagues [9] reported impaired retention of an aversive stimulus up to 3 days post training in mice that were SD prior to training while deficits were only significant 10 days post training when SD was occurring following training. This suggests timely effects of SD on memory consolidation and retrieval.

Similar to observation in the Y maze, spatial memory performance in the Morris water maze was improved by ASD. The probe trial also revealed increased time spent in quadrants 1 and 3 where the platform had been located compared to the other two quadrants in ASD rats. This contrasts with earlier findings that reported impaired memory acquisition following 4 h of paradoxical sleep deprivation using a MWM alternation task [29]. Although difficult to reconcile, differences between studies could in part be attributable to prior exposure to passive avoidance task, which may have facilitated memory retrieval of ASD rats in the current study and/or attenuated stress associated with testing. This is supported by the increased time to reach the platform in control and SD rats in the former studies [29,30], considering similar task difficulty. Repeated exposure to 4 h SD had no impact on spatial memory. This is interesting considering that bilateral lesion of the pons, which prevents REM sleep, has no effect on memory processing and consolidation in humans [53,54]. More studies are necessary to assess underlying mechanisms, and the second part of this study aimed to contribute to such efforts.

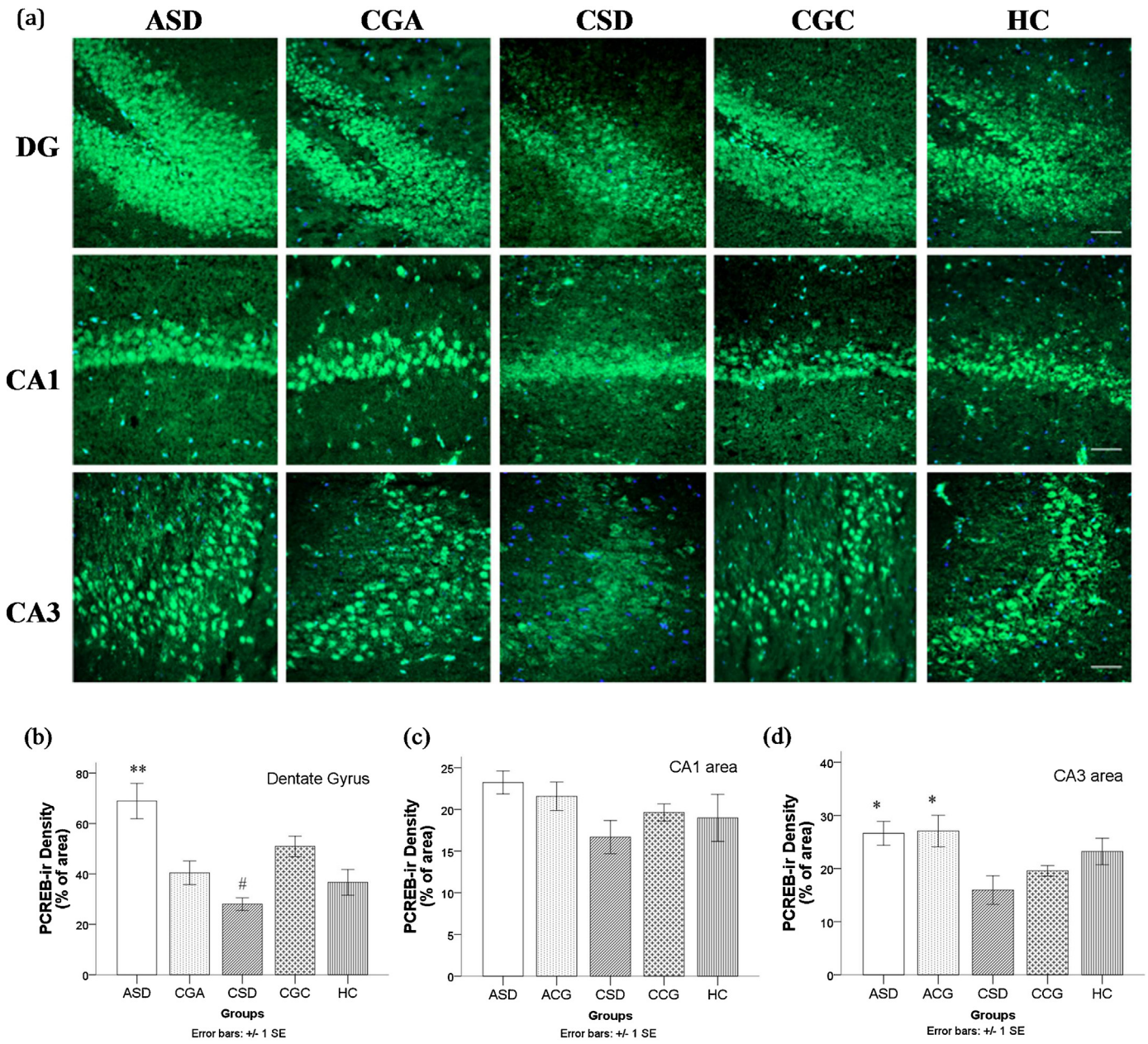

Fig. 9. Effect of sleep deprivation on pCREB-ir at the hippocampal DG, CA1 and CA3 subregions. (a) Representative photomicrographs of pCREB-ir (green) and Hoescht (blue) labeling for the different groups. Scale bar, 50 μm (20× magnification). (b–d) Histograms showing pCREB-ir densities for the different regions. Results are presented as means ± SEM. **Significantly elevated compared to all other groups, $p < 0.01$. #Significantly reduced compared to CGC, $p < 0.05$. (d) *Significantly elevated compared to CSD, $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. Effects of single and repeated SD on GR, TH, pCREB and Ki-67 expression

Different studies have assessed the impact of SD on neurochemical changes coinciding with the end of the deprivation period [35,44]. In the current study, we were interested in assessing lasting effects that acute and repeated SD periods may induce on brain functions 72 h post deprivation. Brain regions selected are part of biochemical systems shown to interact in regulating different physiological responses, including emotions, stress and cognition [35,43–45].

Our findings revealed significant elevations of GR-ir expression at the PVN and hippocampal CA1 and CA3 layers in repeatedly SD rats, suggesting persistent effects on HPA axis activation impacting negative feedback regulation [55]. However, GR-ir measured at this delayed interval was not correlated with memory performance in the MWM and Y maze determined 1 h following SD. Tiba et al. [55] similarly found impaired contextual fear memory measured following 96 h SD period to be independent of glucocorticoid secretion.

Our study also aimed to determine possible interplay between glucocorticoid receptors and catecholamine signaling in the mesolimbic circuitry. Existing studies have shown that tyrosine hydroxylase (TH) is sensitive to periods of RSD [56–59]. Our findings showed a significant increase in TH-ir at the VTA and NAc and DRD1-ir at the lateral hypothalamus (LH) in ASD rats. In contrast, CSD led to reduced DRD1 expression at the LH. GR has been shown to affect dopamine expression [45]. We observed a negative correlation between GR-ir at the PVN and hippocampus and TH-ir at the NAc and VTA, and DRD1-ir at the LH, suggesting that these

neurochemical systems could interact in regulation of SD effects. DRD1- and GR-ir neurons are present in a small subset of neurons in the VTA, which support the possibility of a bi-directional functionality, as well as implying that effects on dopamine synthesis are also likely modulated by mechanisms independent from GR activation [60]. The involvement of the lateral hypothalamus (LH) in sleep regulation is established [61,62], and melanin/fos double-labeled neurons are selectively increased in the lateral hypothalamus after 72 h SD using the platform-on-water a method [63]. Of note, D1 receptor antagonists administered at the lateral hypothalamic level of the medial forebrain bundle blocks the learned conditioned taste aversion [64–66], which may indicate a role for DRD1 activation in enhanced avoidance learning observed in ASD.

These brain regions also share anatomical connections with the amygdala, hippocampus and prefrontal cortex, regulating attention and motivation/reward processes. Via interactions with glucocorticoids, mesolimbic catecholamines also act to modulate physiological adaptation to various stressors and play a role in the negative effects of chronic stressors on cognitive and emotional behaviors [44]. As for diminished TH-ir expression in A11 neurons of the hypothalamus in the CSD rats, effects at this region is likely independent from the interplay with GR, as these neurons are not part of the mesolimbic circuitry but rather project dopaminergic neurons to the spinal cord [67]. This cell group has been associated with waking and/or inhibition of REM sleep during RSD [43].

Cyclic-AMP response element-binding protein transcription factors, including pCREB, are known to play an important role in fostering plasticity of brain systems enabling learning and acquisition of different forms of long-term memory in the hippocampus [68], as well as integration of functional adult-generated neurons into existing neural networks [1]. Our data revealed heightened pCREB-ir in the dentate gyrus and hippocampal CA3 layer following ASD, while expression was reduced following CSD. Within the hippocampus, input from the entorhinal cortex to the dentate gyrus is ramified by the connections between the dentate gyrus and the CA3 pyramidal neurons [69,70]. Adaptive plasticity of the brain has been widely studied in animals exposed to stress and shown to involve different mediators, including NMDA receptor activation by glutamate [70]. Thus, elevation of glutamate levels following acute restraint stress has been linked to increased pCREB activation [69], while prolonged sleep deprivation periods of 12, 24 and 72 h have been shown to reduce expression of glutamate NMDA and AMPA receptors, and impaired synaptic plasticity and cognitive performance [71–73]. In our study, pCREB expression in the DG is negatively correlated with latency in the MWM, while expression in the CA1 and CA3 layers are positively correlated with latency 2 in the YM-PAT. This may indicate that adaptive structural plasticity fostered by ASD could promote learning and memory in situations of moderate arousal.

Finally, Ki-67-ir was used to assess cell proliferation in the DG and subventricular zone (SVZ) along the outer wall of the lateral ventricles. Increased Ki-67 expression in the SVZ was observed in ASD rats. This is an interesting finding considering the proposition that dopamine regulates cell proliferation and neurogenesis in the germinal SVZ [74–77] or is implicated in SVZ function [43]. For example, Lennington and colleagues [43] indicated that dopamine signaling in the SVZ originates from a population of ventrolateral VTA neurons that are more typically associated with motivational and reward processing, but are able to regulate SVZ cell proliferation (see [77] for review). In contrast to acute treatment, repeated SD led to a reduction in Ki-67-ir in the SVZ as compared to that of all other groups. Interestingly, these rats also showed reduced TH- and DRD1-ir in areas of the mesolimbic dopamine circuit, as well as reduced TH-ir in the A11 noradrenaline cells of the hypothalamus. Although these observations remains correlational, such changes could contribute to duration-dependent effects on cell proliferation, consistent with reports showing that dopamine denervation reduces SVZ cell proliferation [75].

Our findings revealed no alterations of Ki-67 expression in the subgranular zone (SGZ) of the DG, consistent with other studies showing no impact on SGZ cell proliferation with shorter than 24 h SD regimen [1,15,17,78,79]. In contrast, SD for periods of 72 h or more led to significant reductions in SGZ cell proliferation [16,80]. Using adrenalectomized and sham operated rats, Mirescu and colleagues [17] showed that suppression of hippocampal cell proliferation upon prolonged SD is in part mediated by elevated glucocorticoid secretion. However, a number of studies have reported altered hippocampal neurogenesis to be independent of adrenal stress hormone secretion [16] (for review see [1]). In sum, sleep may not directly promote cell proliferation and maturation, although it may be essential for normal functioning of other processes and systems (e.g. locomotor activity, hypothalamic–pituitary–adrenal axis activity, and activity of neurotransmitter systems) which in turn regulate cell proliferation and/or neurogenesis [1].

5. Conclusion

This report is the first to evaluate changes in a variety of neurochemical signals induced by either a single or a repeated exposure to short-term sleep deprivation (<24 h), in conjunction with behavioral testing. Our findings support beneficial effects of ASD on memory, while duration-related effects on brain signaling were present 72 h following SD. These findings open avenues for determining whether observed neurochemical changes following SD affect emotional regulation and reward, areas of investigations that remain neglected by existing studies.

Acknowledgments

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